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Ultrathin Coatings with Change in Reactivity over Time Enable Functional In Vitro Networks Of Insect Neurons**

By *Anna Reska, Peter Gasteier, Petra Schulte, Martin Moeller, Andreas Offenhäusser,* and *Juergen Groll**

Cellular processes such as sensing mechanisms and signal transduction have evolved to be highly specific and complex. In order to take advantage of these processes, artificial environments must be designed to allow for precise control over cell behavior and exploitation of cell functions. For example, technological applications such as implantable devices and biosensors can be engineered using biomimetic approaches.[1–4]

Combining a suitable microfabrication technology with surface modification techniques provides adherent binding sites for growing cells in spatially controlled regions with a nonfouling background.[5] Such biofunctional patterns on a protein-repellent background yield geometrically confined cellular networks on artificial surfaces. This in vitro technique provides a means of systematic manipulation of the extracellular environment for investigating cell–surface and cell–cell interactions. Covalent immobilization of extracellular factors also provides pattern stability for long-term cell-based experiments.[6]

Most attempts to covalently link patterned cell adhesive molecules to substrates with a nonadhesive background rely on multistep procedures.[5,7,8] Microfabrication techniques, such as photolithography, ink-jet technology, microfluidics, and microcontact printing (μCP), have been used for generating patterns of cells on artificial substrates.[9,10] μCP is an especially valuable technique for surface pattern generation[11,12] owing to freedom in geometric pattern design, down to the nanometer scale, and diversity in solutions that can be used as "ink". Beyond spatial control, successful cellular patterning on artificial substrates also requires precise control over surface chemistry. One of the core challenges in the design of biosensors and biohybrid devices is the development of universal strategies for covalently attaching biomolecules to solid-state components.[13]

Here, we present a coating system that combines nonfouling properties[14] with a single-step covalent coupling scheme for attachment of biomolecules without further use of chemical crosslinkers. Ultrathin hydrogel layers of isocyanate-functionalized starshaped prepolymers, based on poly(ethylene glycol), change chemical reactivity from amino-reactive, during layer preparation, to cell-repellent, after hydrolysis of the isocyanate groups. We demonstrate the applicability of this system to biosensor technologies and biohybrid devices by geometrically controlling specific adhesion factors and growth of functional insect neuronal networks. Neurons are used for biosensing devices because they use electrical signals for task-related information processing. When coupled to electronic devices, this signaling can be used for sensing. Here we used neurons from the terminal ganglion of crickets, *Gryllus bimaculatus*. These neurons control the highly sensitive, wind-evoked escape reaction of crickets and represent simple, functional networks for enhancing the understanding of neuronal processing. The extraordinary ability of these cells to adhere to various artificial substrates has been an obstacle to directing network formation so far. By using the surface coating presented here, in vitro formation of functional, patterned insect neuronal networks is shown for the first time.

Surface coatings were prepared from aqueous solutions of six-armed starshaped poly(ethylene glycol)-based prepolymers terminated with isocyanate functional groups (star-PEG). Figure 1 displays the chemical structure of the system and the reaction that is initiated upon contact of the material with water. Hydrolysis of the isocyanate groups is the first step in a crosslinking process that forms a dense polymer film. First, the isocyanate groups react with water to carbaminic acid groups, which are not stable at neutral pH and instantly decarboxylate to amino-groups. These amino groups then react with remaining isocyanate groups to form urea linkages between two star prepolymer arms. This process is accompanied by a change in functionality from isocyanate to urea bridges between the star molecules and remaining free amino groups over time. To ensure the long-term stability of thin star-PEG films on substrates such as glass or silicon, the polymer can be tightly linked to the support by aminosilanization of the

[*] Dr. J. Groll, P. Gasteier, Prof. M. Moeller
DWI e.V. and Institute of Technical and Macromolecular Chemistry, RWTH Aachen University
Pauwelsstr. 8, 52074 Aachen (Germany)
E-mail: groll@dwi.rwth-aachen.de
A. Reska, Dr. P. Schulte, Prof. A. Offenhäusser
Institute for Bio and Nanosystems (IBN–2), Forschungszentrum Jülich
52425 Jülich (Germany)

[**] A.R. and P.G. contributed equally. The authors want to thank Dr. Krystyna Albrecht for SFM measurements and Dr. Kristin E. Michael for evaluation of the manuscript. The German Science Foundation (DFG, graduate school 1035 "Biointerface", Project B1 in the TR-SFB 37 and SPP 1259 "Intelligent Hydrogels") and the EU (project "CILIA", contract 016039, www.cilia-bioincs.org) are acknowledged for funding. SusTech GmbH & Co KG is acknowledged for support with the coating technology. Supporting Information is available online from Wiley InterScience or from the authors.

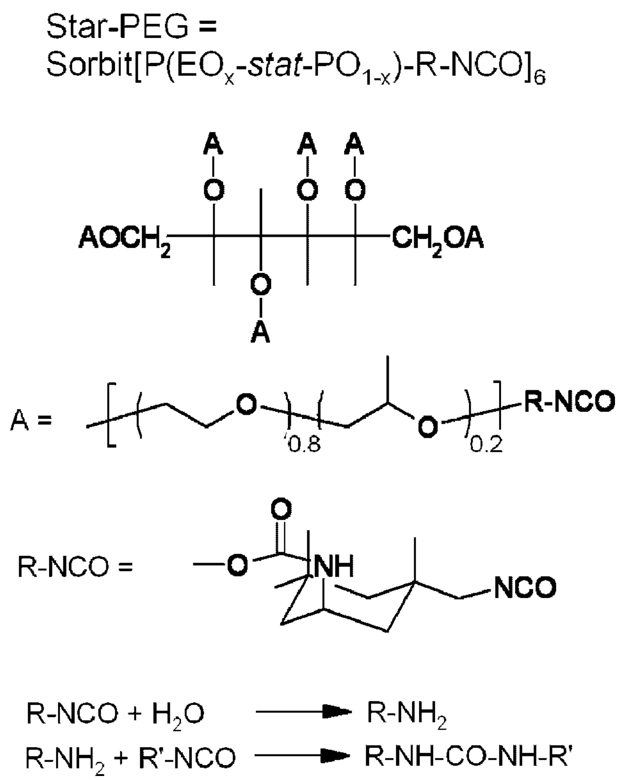

Figure 1. Chemistry of the star-PEG system and the reactions that lead to film formation. The backbone consists of statistically polymerized ethylene oxide and propylene oxide in a ratio of 4:1. The reactive isocyanate end groups hydrolyze in aqueous environment to amino groups that, in a following step, react with isocyanate groups to urea bridges between two stars.

substrate prior to coating. Here, we used silicon wafers and glass slides treated with N-[3-(trimethoxylsilyl)propyl] ethylenediamine. Nonfunctionalized star-PEG layers prevent unspecific protein adsorption and the adhesion of several human cell types under standard cell culture conditions over weeks of exposure.[15]

Because not all isocyanate terminal groups are hydrolyzed instantly upon contact with water, a portion of them will remain unreacted and are available for further functionalization in freshly prepared films.[16] This can be used to present specific cell-adhesion molecules to control cell attachment and growth. Remaining isocyanate groups can be used to bind amino- or hydroxyl-functional compounds such as low-molecular-weight binding ligands or complete proteins through binding of peripheral lysines. Owing to the higher nucleophilicity of amines, the aminolysis of isocyanates is generally some orders of magnitudes faster than the hydrolysis or even alcoholysis.[17] Thus, hydrolysis of the isocyanate groups to amino functional groups is the rate-limiting step of the crosslinking reaction within the star-PEG film. This limiting step provides a time window of several hours in which the films bear isocyanate groups that are available for covalent binding of amino-functional compounds. We used this time window for covalent patterning of lectin concanavalin A (conA) on freshly prepared films. This protein supports the adhesion of insect neurons.[18]

Patterning of conA was achieved by means of μCP.[19,20] A PDMS stamp provided a rectangular grid pattern of 6 μm lines with side lengths of 50 and 100 μm. Each intersection had a node of 20 μm in diameter designed to capture cell bodies of cultured cricket neurons. At ambient conditions, the star-PEG needed a minimum crosslinking time of 1 h after coating to reach enough mechanical stability, such that the printing procedure did not result in mechanical deformation and topological structuring of the films. However, owing to the crosslinking within the film the concentration of isocyanate groups decayed with time. Therefore, μCP was performed at various time intervals after coating. Protein immobilization and pattern formation without mechanical deformation was best when the stamping was performed 2 h after coating (determined by scanning force microscopy (SFM) and scanning electron microscopy (SEM); see Supporting Information Fig. S1) and was used as the standard procedure for all further experiments.

The stability of the printed micropattern was tested by washing experiments. When conA was stamped onto fully crosslinked star-PEG films, the weakly physisorbed proteins could be washed off, whereas the conA that was brought in contact with the star-PEG as described above could not be washed, indicating covalent binding of the protein to the layer (Supporting Information Fig. S2). The SEM measurement showed a smooth star-PEG surface on which a conA grid pattern was deposited by μCP (Supporting Information Fig. S1B). This substrate had been used in cell culture for 2 days before imaging by SEM, further demonstrating the stability of our surface-coating system and the micropatterns under cell-culture conditions.

Both the SEM and the SFM images also revealed that more protein was deposited at the edge of the structure compared to the middle of the nodes and lines. Since the protein is transferred from aqueous solution to the hydrophobic PDMS stamp by incubation, partial dewetting may occur during drying of the stamp, resulting in accumulation of proteins at the edges of the stamp structure. Hence, the rim of the structure transfers more protein.

The conA transfer to the star-PEG surface was continuous over large sample areas, as determined by stamping rhodamine red and fluorescein isothiocyanate (FITC)-labeled conA and imaging with confocal laser scanning microscopy (Fig. 2). Although there were variations of protein density within the pattern, the pattern was transferred continuously over large surface areas. Corresponding results have been obtained by ellipsometry mapping of conA-structured surfaces (Supporting Information S3).

Studies on vertebrate neurons have shown geometrically controlled adhesion and mimicking of neuronal networks on artificial surfaces.[21,22] In contrast, only few studies[23] have been published that deal with insect neurons, although these cells are highly promising candidates for the formation of task-related functional and biomimetic neuronal networks on

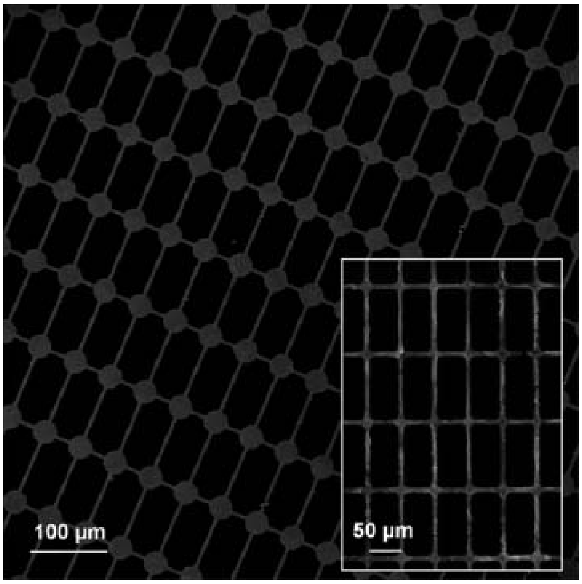

Figure 2. Confocal laser scan image of rhodamine-red-labeled conA printed on a star-PEG surface (line width 5 μm, nodes width 20 μm; grid pattern 50 × 100 μm^2). The inset shows printed FITC-labeled conA (line width: 6 μm; nodes width: 14 μm; grid pattern 50 × 100 μm^2).

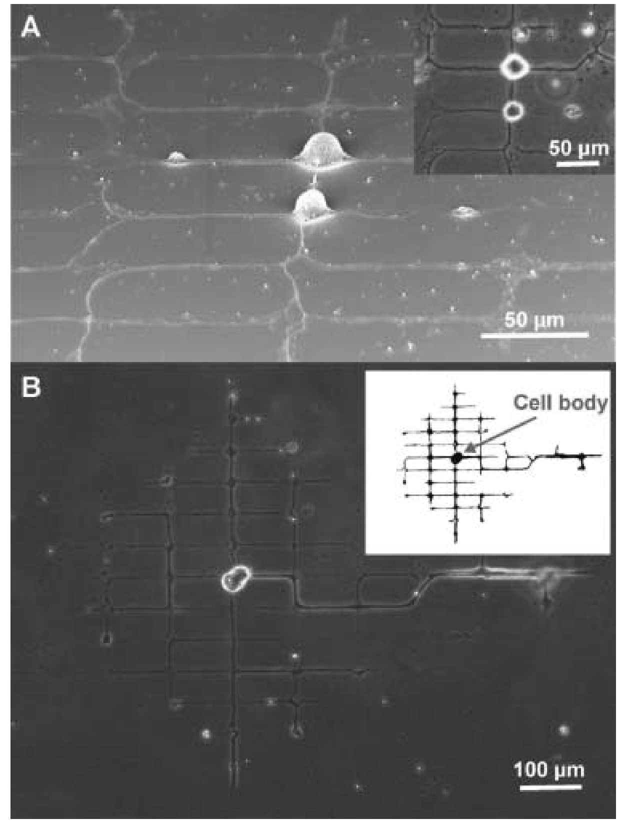

Figure 3. Pattern fidelity of cricket neurons grown on a conA/star-PEG surface after A) 8 and B) 10 days in culture, visualized by SEM (A) and phase-contrast light microscopy (B). The inset in (A) shows the same 2 cells before sample preparation for SEM in cell culture by phase-contrast light microscopy. Line width: 6 μm, node width: 25 μm, node distance: 50 μm and 100 μm.

artificial substrates. Sensing mechanisms in insects are controlled by small but highly specialized nervous systems. The neuronal tissue consists of a low number of cells. Hence, the number of neurons involved in the circuitry for signal processing is small. For example, during the wind-evoked escape reaction of crickets the signals are propagated to other centers of the brain by only 20 interneurons.[24] In cell culture, these neurons are easy to handle and stable against environmental changes. They form simple but well working in vitro neuronal networks. Thus, the approach to growing a small number of cricket neurons in geometrically controlled patterns is a highly attractive alternative to generating biomimetic neuronal networks on artificial surfaces. Patterned adhesion and growth of insect neurons enables the study of how cellular circuits of defined cell populations transfer and process information. Moreover, analytical devices that employ insect neurons combine sensitivity with robustness. Insect cells are also highly adhesive to various surfaces. In order to take advantage of the cellular network, cell adhesion and neurite outgrowth has to be precisely controlled and directed by an appropriate surface modification. Thus far, this prerequisite has been the predominant reason why geometrically controlled network formation of insect neurons on artificial surfaces could not be achieved.

Neurons of crickets' terminal ganglia were seeded onto prepatterned surfaces after full crosslinking of the star- PEG films according to methods described previously.[18,25] The cells did not adhere onto the unmodified star-PEG layers, but adhered preferentially to the conA grid pattern and showed first neurites within 12 h (data not shown). SEM and phase contrast microscopy images of cricket neuronal cells revealed precise geometrical restriction of the neurite growth to the conA modified surface (Fig. 3). Neurites often followed the structure at the edge of the patterned lines (see nodes in Fig. 3A) in accordance with the higher amount of deposited protein at the edges. The neurite length extended over several hundred micrometers resulting in tremendous neurite trees (Fig. 3B). The neurons conserved this distinct morphology for up to 3 weeks in cell culture indicating an outstanding stability and biocompatibility of the surface modification. The presented surface modification operated effectively for very large surface areas, as documented by phase-contrast microscopy over square-millimeter-sized surface areas (Supporting Information Fig. S4). Slight variations of protein density in the overall homogeneous protein pattern documented by fluorescence microscopy did not significantly affect the adhesion of the cricket neuronal cells.

Besides the generation of a defined morphology the cricket neurons also retained function, as evidenced by electrical activity. Whole-cell patch-clamp recordings showed that membrane potential, amplitude, and frequency of action potentials (APs) for patterned neurons were in good correspondence to APs for both in vivo and unpatterned in vitro cricket neurons

ADVANCED MATERIALS

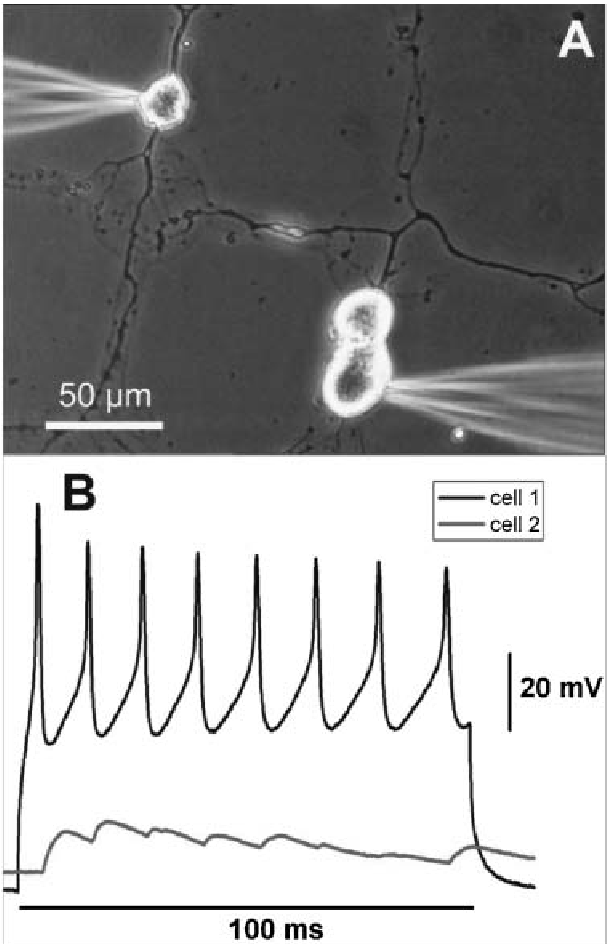

Figure 4. A) Phase-contrast light microscopy of double patch-clamp recording obtained from a morphologically connected pair of 2 cricket neurons after 5 days in culture on a conA/star-PEG surface. The image shows the exact position of the patch-clamp pipettes during the recording (upper left and lower right). B) Stimulation of the presynaptic cell led to action potentials in this cell (black, top line) followed by correlated and summated postsynaptic potentials in the postsynaptic cell (grey, lower line), although the neuronal cell bodies have a distance of more than 100 μm, indicating that the synaptic contact is localized on the neurites. The synaptic latency was approximately 1 ms, which is a typical value for a chemical synapse.

(data not shown). Double patch-clamp recordings revealed cell–cell communication between pairs of morphologically connected neurons via electrical signals (Fig. 4). Chemical synapses mediate unidirectional signal propagation between two adjacent neurons via an asymmetric interface. The membranes of the pre- and postsynaptic neurons at this interface contain different molecular components and complexes. Triggered by a change in membrane voltage, for example APs, the presynaptic neuron secretes signaling molecules (neurotransmitters) into the interface, which bind to receptors of the postsynaptic neuron. The open probability of nearby ion channels in the postsynaptic cell membrane is altered and results in a voltage change (postsynaptic potential, PSP).[26] These signals were recorded by measuring the membrane potential of two connected cells within the network simultaneously, while one of the neurons was stimulated by defined current pulses generated by an operational amplifier. Stimulation of the upper neuron failed to elicit a response in the adjacent neuron (data not shown). However, stimulation in opposite direction resulted in presynaptic APs (black line) of the lower neuron triggering graduated postsynaptic potentials (PSPs, grey line) in the upper neuron. The first two APs of the lower neurons produced PSPs with amplitudes up to 10 mV in the upper neuron, while the response from the following APs was possibly masked by postsynaptic repolarization. Hyperpolarizing presynaptic stimuli did not affect the postsynaptic neuron (data not shown). The synaptic delay between the peak of the presynaptic AP and begin of PSP was approximately 1 ms, which is in the typical range for chemical synapses. The double patch-clamp recordings demonstrate the existence of functional synapses in the patterned cricket neuronal networks. These results also emphasize the suitability of the surface modification to control the growth of neuronal networks and excellent biocompatibility of the star-PEG system.

In conclusion, this study presents a powerful and universal surface modification technique for the generation of specifically interacting cell culture substrates and biosensors. Biofunctional molecules can be directly introduced without the need for further process steps or chemical crosslinkers owing to a unique intrinsic property of this material, in which reactivity changes from highly reactive isocyanate groups into nonreactive and cell-repulsive. This feature enables binding of both amino and alcohol groups, facilitating coupling of various molecules to our material. The applicability of this system to biosystems is demonstrated by the development of a reliable method for the geometric growth control of cricket neuronal cells through simple microcontact printing and covalent binding of conA onto star-PEG films. In cell culture, the insect neurons regenerated tremendous neurite trees exclusively along the protein micropattern. Patch-clamp recordings evidenced that the surface modification did not affect the electrophysiological parameters of the neurons. Moreover, we demonstrate the first direct correlation between presynaptic stimulation and postsynaptic events in a patterned insect neuronal cell culture. These results are essential for the construction of defined insect neuronal networks in vitro. The broad applicability of the reactivity change from reactive isocyanate groups to an amino-group-containing cell-repellent coating makes star-PEG a highly effective and versatile coating system for applications that require the interfacing of biological molecules with artificial surfaces such as biosensors, cell-based assays, and implantable devices.

Experimental

Substrate Preparation and Star-PEG Film Formation: Samples have been prepared as previously reported [14,16]. Briefly, glass and silicon substrates were cleaned by ultrasonication (acetone, water, and isopropyl alcohol; 1 min each), dried in a stream of nitrogen, and activated by a UV/ozone treatment (12 min). Directly after activation, substrates were incubated in a solution of *N*-[3-(trimethoxysilyl) propyl] ethylenediamine (0.2 mL) in dry toluene (50 mL) for 2 h in an inert gas atmosphere. The samples were then washed several times with dry toluene and dried in a stream of nitrogen prior to spin-coating. For preparation of the star-PEG film, isocyanate-terminated prepolymer (100 mg; $M_W = 12$ kDa, polydispersity (PD) = 1.2) was dissolved in dry tetrahydrofuran (1 mL). After addition of demineralized water (9 mL), this solution was gently stirred for 5 min, completely wetting the substrate. Spin-coating was performed at 2500 rpm for 40 s (R60, Convac). These freshly prepared films were stored in ambient conditions for 2 h prior to μCP. The parameters chosen for the casting process in this study resulted in star-PEG coatings with a thickness of 30 nm. Layer thicknesses were determined using a Nanofilm EP3 ellipsometer with a Nd:yttrium aluminum garnet (Nd:YAG) laser (532 nm) and EP3 View 2.05 software. The angle of incidence was 70°.

Microcontact Printing: Polydimethylsiloxane (PDMS) stamps with grid patterns (line width: 6 μm, node width: 20 μm, node distance: 50 μm and 100 μm) were fabricated by pouring Sylgard 184 (Dow Corning, Midland, MI, USA) onto silicon masters (fabricated by standard lithographic procedures as described elsewhere [19]) followed by a 12 h curing step at 80 °C. Final curing, after master stamp release, was performed for 1 h at 110 °C. The stamps were inked by immersion in a conA solution (0.5 mg in 1 mL purified water) for 10 min, dried in a stream of nitrogen, and placed onto the star-PEG coating for 10 min.

Patch-Clamp Recordings: Whole-cell patch-clamp experiments were performed as described previously [22]. The culture medium was replaced by a normal salt solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 10 mM HEPES; ca. 390 mOsm kg^{-1} adjusted by glucose and pH 7.0 by NaOH). Pipettes were pulled from borosilicate glass filaments (o.d. 1.5 mm, i.d. 0.86 mm; Sutter Instrument Co., Novato, CA, USA) using a Flaming/Brown micropipette puller (P-97; Sutter Instruments Co., Novato, CA, USA) resulting in a pipette electrode resistance of 4–11 MΩ. Glass pipettes were filled with internal solution (8 mM NaCl, 180 mM KCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Na_2-ATP, 1 mM EGTA and 10 mM HEPES; ca. 390 mOsm kg^{-1} adjusted by equal shares glucose/fructose and pH 7.0 by NaOH). Whole-cell patch-clamp recordings were made using an EPC9/3 amplifier controlled by TIDA 5.x software (both HEKA Elektronik, Germany). The data were sampled at 25 kHz and filtered at 10 kHz with a Bessel filter. The liquid-junction potential was calculated using JPCalcW 1.0 software (P.H. Barry, University of New South Wales, Sydney, Australia and Axon Instruments Inc., CA, USA) subtracted from the measured membrane voltages. Electrophysiological experiments were performed under optical control (Olympus IX 50 inverted microscope equipped with phase-contrast). All experiments were performed at room temperature.

Cell Fixation for SEM: For fixation, the cell-culture medium volume was substituted gradually with glutaraldehyde (3.5% in 20 mM HEPES, adjusted to pH 7.0 with NaOH) and incubated over night at 4 °C. To dehydrate the cell material, ethanol (p.A. grade) was added and the concentration was gradually raised up to approximately 100%. After critical-point drying in ethanol, the substrates were sputtered with gold for 30 s at 50 mA under vacuum.

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